REMARKS/ARGUMENTS

In response to the Office Action of January 4, 2006, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Claim Status/Support for Amendments

New claims 15-17 have been added. Claims 1-4 have been cancelled. Claims 13 and 14 were cancelled in a previous response (filed on June 27, 2005). Claims 5-12 are withdrawn from consideration. It is understood that claims 5-12, drawn to the non-elected invention, will remain pending, albeit withdrawn from consideration on the merits at this time. Applicants wish to preserve their right to present claims 5-12 in a divisional application(s).

Originally examined claims 1-4 are cancelled herein. Claims 15-17 find their basis in and replace claims 1-4, thus, claims 15-17 are currently under examination. Claims 5-12 and 15-17 remain pending in the instant application.

No new matter has been added by the addition of new claims 15-17.

New claim 15 finds its basis in claim 1 as originally filed and clarifies that the claimed method can be used for diagnosing Alzheimer's dementia by determining the presence of a

thrombospondin polypeptide weighing about 180 kDa in a sample of body fluid. This method finds support throughout the instant specification as originally filed, see, for example, page 1, lines 5-14; page 10, line 12 to page 11, line 1; page 15, lines 6-15; page 19, line 22 to page 21, line 13 and Figures 1 and 2.

New claims 16 and 17 are original claims 3 and 4 rewritten for proper dependency on claim 15.

Rejections under 35 USC 112, first paragraph

Claims 1-4, as presented on June 27, 2005, stand rejected under 35 USC 112, first paragraph, because the specification, while being enabling for detection of thrombospondin, allegedly does not reasonably provide enablement for diagnosis of dementia via detection of a marker indicative of thrombospondin or of thrombospondin in body fluid from mammalian samples. The Examiner alleges that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

The Examiner asserts that the specification is insufficient to enable one skilled in the art to practice the invention as broadly claimed without undue experimentation. The factors relevant to this discussion include the quantity of experimentation

necessary, the lack of working examples, the unpredictability of the art, the lack of sufficient guidance in the specification and the breath of the claims.

The Examiner asserts that Applicants claims are directed to diagnosis of dementia via detection of a marker indicative of thrombospondin or of thrombospondin in body fluid from mammals.

The Examiner alleges that the specification does not enable the broad scope of the claims because the specification fails to delineate those markers that are suitably indicative of thrombospondin besides detection of thrombospondin itself. Further, the specification fails to delineate any suitably correlative marker, thrombospondin or otherwise that is indicative of the diagnosis of dementia.

The Examiner asserts that the art recognizes that even today definitive diagnosis of dementia as a condition in and of itself is confounding because of mental decline that may be linked to a plethora of diseases and/or conditions. For example, dementia is associatively developed in patients with the clinically distinct pathologies of Down's syndrome, Parkinson's disease, Huntington's disease, vascular disease or stroke as well as Alzheimer's disease. Cognitive decline is also recognized among normal aged patients. The closet diagnostic measure is that as delineated in DSM criteria, but is not definitively associated as a singular disease

or disease state, see in particular de Mendonca et al. (Journal of Molecular Neuroscience 23(1-2):143-148 2004); Misciagna et al. (International Journal of Neuroscience 115(12):1657-67 2005) ; Kilada et al. (Alzheimer's Disease Assoc. Disord. 19(1):8-16 2005) and The Rowland Universal Dementia Assessment Scale (RUDAS): a multi-cultural cognitive assessment scale. Even so, the specification fails to distinguish correlation of any particular marker with any art accepted measure that is indicative and correlative to dementia diagnosis. Accordingly, no basis is provided that thrombospondin or thrombospondin markers are diagnostically indicative of any particular disease state or particular to dementia. While thrombospondin is recognized as being present in Alzheimer's plaque pathology and normal brain, see in particular Buee et al. (American Journal of Pathology 141(4)783-788 1992), such is not diagnostic to dementia. Moreover, thrombospondin is noted to be detected in biological mammalian fluids not associated with dementia conditions, see for example Clezardin et al. Journal of Chromatography 296:249-256 1984) and Lawler et al. (Blood 67(2):555-558 1986). This raises the issue that detection of thrombospondin or thrombospondin markers in blood are not definitive of dementia and would provide reference or sample testing subject to false positive diagnosis.

The scope of the claims must bear a reasonable correlation with the scope of enablement (*In re Fisher* 166 USPQ 1924 (CCPA 1970)). Without such guidance, the changes which can be made and still maintain activity/utility is unpredictable and the experimentation left to those skilled in the art is unnecessarily, and improperly, extensive and undue. See *Ex parte Forman*, 230 USPQ 546 (Board of Patent Appeals and Interferences 1986). Thus, the skilled artisan cannot readily make and use the claimed sequences without further undue experimentation.

Applicants respectfully disagree with all of the Examiner's assertions.

Claims 1-4 have been cancelled and new claims 15-17 have been added. New claims 15-17 are drawn to a method for diagnosing Alzheimer's dementia by determining the presence of a thrombospondin polypeptide weighing about 180 kDa in a sample of body fluid. Figures 1 and 2 provide data which evidences that a thrombospondin polypeptide weighing about 180 kDa was identified in samples obtained from patients having Alzheimer's disease but was not identified in samples obtained from patients age-matched with the patients having Alzheimer's disease. Thus, the 180 kDa thrombospondin polypeptide is shown to be a marker of Alzheimer's dementia and it follows that the instant specification as originally filed fully enables a method of diagnosing Alzheimer's

dementia by determining the presence of a thrombospondin polypeptide weighing about 180 kDa in a sample.

Accordingly, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.

Claims 1-4, as presented on June 27, 2005, stand rejected under 35 USC 112, first paragraph, as containing subject matter which allegedly was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time that the application was filed, had possession of the claimed invention.

The Examiner asserts that the specification is on point to the protein thrombospondin, which is asserted as being diagnostic to dementia. However, the claims as written are directed to the term "a marker indicative of thrombospondin". However, the specification fails to delineate markers that are "indicative of thrombospondin". The specification provides the following definition with respect to "marker" at pages 10-11. "As used herein the term "marker" "biochemical marker" or "marker protein" refers to any enzyme, protein, polypeptide, peptide, isomeric form thereof, immunologically detectable fragments thereof, or other molecule, whose presence, absence or variance in fluids from so-called "normal" levels, are circulating bodies indicative of dementia. Most particularly, such markers may be illustrated as being

released from the brain during the course of dementia related changes, e.g. AD pathogenesis. Such markers include, but are not limited to, any unique proteins or isoforms thereof are particularly associated with the brain. Yet such does not describe any member protein that is suitably "indicative of thrombospondin" and diagnostic to dementia.

A genus claim may be supported by a representative number of species as set forth in Regents of the University of California v. Eli Lilly & Co, 119F3d 1559, 1569, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997). For example, a description of a genus of cDNAs may be achieved by means of a recitation of representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus, or recitation of structural features common to the genus, which features constitute a substantial portion of the genus.

The Examiner asserts that the instant specification discloses, however, a single polypeptide thrombospondin that is asserted to be diagnostic to dementia but provides no evidence for such a correlation of for the correlation of any particular marker indicative of thrombospondin that provides for diagnosis. Accordingly, the scope of the genus recitation lacks evidence of even a single species member. Given the unpredictability of diagnostic correlative comparisons, and the fact that the

specification fails to provide objective evidence of any species of the claimed genus it cannot be established that a representative number of species have been disclosed to support the genus claim. No correlative structure or functional activity for the marker is set forth. Accordingly, the Examiner concludes that the claims lack adequate written description support.

Applicants respectfully disagree with the Examiner's conclusion.

The term "marker" as used in the instant specification refers to any molecule whose presence, absence or variance, i.e. differential expression, from levels in a normal physiological state is indicative of a disease state. For example, in the instant case, a 180 kDa protein is found to be present in samples obtained from Alzheimer's disease patients and absent in samples obtained from patients age-matched with the Alzheimer's disease patients. Thus, this 180 kDa protein is considered to be a "marker" of Alzheimer's disease.

Thrombospondin-1 (TSP-1) is known to be a trimeric protein composed of three disulfide-linked 180 kDa polypeptide subunits (see page 3 of the attached article by Abdeloushed et al. as accessed from jbc online on June 28, 2006; Journal of Biological Chemistry 275(24):17933-17936 2000; reference 1). The instant inventors have identified the 180 kDa protein as a polypeptide

subunit of thrombospondin-1 (see the instant specification at page 19, line 22 to page 21, line 13 and Figures 1 and 2). Since the quaternary structure of thrombospondin-1 is known to be made up of an arrangement of three poylpeptide chains each weighing about 180 kDa; one of the polypeptide chains is considered indicative of the whole thrombospondin-1 protein. Thus, the instant inventors refer to the protein identified in the described experiments as a "marker indicative of thrombospondin".

Furthermore, autoantibodies against thrombospondin are also contemplated as "markers indicative of thrombospondin" (see the instant specification at page 15, line 16 to page 16, line 5).

Thus, Applicants respectfully submit that, contrary to the Examiner's assertion, they had possession of "markers indicative of thrombospondin" at the time that the instant application was filed. However, in the interest of compact, efficient prosecution, claims 1-4 have been cancelled and new claims 15-17 recite "a thrombospondin polypeptide weighing about 180 kDa".

Accordingly, Applicants respectfully request that this rejection under 35 USC 112, first paragraph, now be withdrawn.

Rejection under 35 USC 112, second paragraph

Claims 1-4, as presented on June 27, 2005, stand rejected under 35 USC 112, second paragraph, as being indefinite for

allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner notes that the claims are directed to the term " a marker indicative of thrombospondin". However, the Examiner asserts that the specification fails to delineate markers that are indicative of thrombospondin. The specification provides the following definition with respect to "marker" at pages 10-11. "As used herein, the term "marker" "biochemical marker" or "marker protein" refers to any enzyme, protein, polypeptide, peptide isomeric form thereof, immunologically detectable fragments thereof, or other molecule, whose presence, absence, or variance in fluids from so-called "normal" levels, are circulating bodies indicative of dementia. Most particularly, such markers may be illustrated as being released from the brain during the course of dementia related changed, e.g. AD pathogenesis. Such markers include, but are not limited to, any unique proteins or isoforms thereof that are particularly associated with the brain. However, such does not clarify whether any brain-associated protein exhibiting such modulation is appropriate or whether particular ones are intended. Accordingly, the Examiner concludes that the scope of the claims cannot be determined.

Applicants respectfully disagree with the Examiner's conclusion. The meaning of the term "marker indicative of

thrombospondin" is clarified above in the response to the rejection under 35 USC 112, first paragraph (written description).

However, in the interest of compact, efficient prosecution, claims 1-4 have been cancelled and new claims 15-17 recite "a thrombospondin polypeptide weighing about 180 kDa".

Accordingly, Applicants have now clarified the metes and bounds of the claims and respectfully request that this rejection under 35 USC 112, second paragraph be withdrawn.

Rejection under 35 USC 102(e)

Claims 1-4, as presented on June 27, 2005, stand rejected under 35 USC 102(e) as allegedly being anticipated by Ni et al. (US 6,605,592 or in the alternative Ni et al. US PGPub 2002 0068319). The Examiner asserts that these references are cumulative and are therefore cited together with identical reasoning therefore.

The Examiner asserts that Ni et al. teach the peptide of thrombospondin and variants that are used for detection of dementia based on expression in platelets and in brain, see in particular columns 22-93 of the patent. In addition, as noted in the PGPub, the invention includes antibodies to the proteins and methods of detection from mammalian samples. In particular, a method of diagnosing a pathological condition or a susceptibility to a pathological condition in a subject comprising: (a) determining the

presence or amount of expression of the polypeptide of claim 11 in a biological sample; and (b) diagnosing a pathological condition or a susceptibility to a pathological condition based on the presence or amount of expression of the polypeptide, is included (see claim 19).

Accordingly, the Examiner concludes that the teachings of Ni et al. fairly anticipate a method for diagnosing dementia in a mammal via obtaining body fluid samples, detecting thrombospondin via antibodies and correlating the presence with dementia, including Alzheimer's.

Applicants respectfully disagree with the Examiner's conclusion.

Claims 1-4 have been cancelled and new claims 15-17 recite "a thrombospondin polypeptide weighing about 180 kDa".

It is well established that in order for a claim to be anticipated each and every element as set forth in the claim must be found, either expressly or inherently described in a single prior art reference (see MPEP 2131).

In general, Ni et al. are concerned with secreted human proteins, and disclose multiple novel human secreted proteins and nucleic acids encoding the proteins. At column 22 of US 6,605,592, the involvement of the thrombospondin family of proteins, including TSP-1, with angiogenesis is discussed. Ni et al. state that there

is a need in the art to identify other molecules involved in the regulation of angiogenesis. Figure 4 of the patent shows the nucleotide sequence (SEQ ID NO:18) and the deduced amino acid sequence (SEQ ID NO:89) of THRAP. The novel THRAP protein exhibits TSP-1-like domains, IgG-like domains and proteinase inhibitor-like domains also shown in Figure 4. Figure 5 shows the regions of identity between the amino acid sequence of THRAP and the translation product of thrombospondin-like protein as determined by BLAST analysis. Thus, Applicants respectfully assert that Ni et al. does not teach a thrombospondin peptide, but a peptide that shares some similar domains with thrombospondin.

Furthermore, even if Ni et al. did disclose thrombospondin, they do not teach a polypeptide subunit of thrombospondin-1 weighing about 180 kDa that was identified as present in Alzheimer's patients and absent in control patients and thus useful as a marker for diagnosing Alzheimer's disease.

Thus, Ni et al. can not be said to either expressly or inherently describe each and every element in the claims as now presented for examination.

Accordingly, Applicants respectfully submit that they have now pointed out how the method as instantly claimed distinguishes over the method taught by Ni et al. and respectfully request that this rejection under 35 USC 102(e) now be withdrawn.

Rejection under 35 USC 103 (a)

Claims 1-4, as presented on June 27, 2005, stand rejected under 35 USC 103(a) as allegedly being unpatentable over Ni et al. (US 6,605,592) or in the alternative Ni et al. (US PGPub 2002 0068319), further in view of Bueé et al. (American Journal of Pathology 141(4):783-788 1992; IDS) and WO 98/07035 (IDS).

The Examiner asserts that WO 98/07035 teaches direct immunoassay amongst blood samples for the presence of thrombospondin, see in particular the abstract, claims and throughout thereby teaching steps (a), (b) and (c).

The Examiner states that the teachings of Ni et al. are as noted above in the 102(e) section. Ni et al. note that dementia may be detected via such analysis for thrombospondin in blood, but fail to teach the direct measurement or correlation of the presence of thrombospondin in blood with the occurrence of dementia.

The Examiner asserts that Bueé et al. note that thrombospondin is part of a family of adhesive glycoproteins and is involved in a number of physiologic processes such as angiogenesis and neurite outgrowth. Immunohistochemical localization of thrombospondin in normal human brains was investigated in the hippocampus and inferior temporal cortex. Two antibodies (one polyclonal and one monoclonal) against thrombospondin-labeled microvessels, glial cells, and a subpopulation of pyramidal neurons. The distribution

of thrombospondin staining in patients with Alzheimer's disease was found comparable to control subjects. However, in patients with Alzheimer's disease a subset of pyramidal neurons that may be vulnerable in Alzheimer's disease exhibited decreased staining. This decrease in the intensity of labeling might constitute a marker for neuronal population prone to early degeneration. In addition, thrombospondin staining was demonstrated in senile plaques in Alzheimer's disease. These results suggest that thrombospondin may be involved in the process of neuronal degeneration and senile plaque formation. Accordingly, Bueé et al. evidences the correlation of the presence of thrombospondin with the occurrence of dementia, even though the correlation was not in blood fluid, this is not a limitation of the claims. In short, thrombospondin detection in blood was known in addition to its presence in brains of demented mammals, particularly within Alzheimer's patients.

Accordingly, the Examiner asserts that the cumulative reference teachings motivate the artisan to diagnose dementia via detection of the correlation of thrombospondin with the occurrence of dementia and motivates the artisan to detect thrombospondin or thrombospondin variants via immunoassay from blood in correlation with its presence in brain pathology associated with Alzheimer's disease and dementia patients, thereby diagnosing dementia.

Accordingly, the Examiner concludes that the cumulative references render the claimed invention obvious to the artisan and establish an expectation of success as Bueé et al. evidences that the dementia patients are in fact correlated with the presence or detection of thrombospondin in blood via immunoassay.

Applicants respectfully disagree with the Examiner's conclusion.

Claims 1-4 have been cancelled and new claims 15-17 recite "a thrombospondin polypeptide weighing about 180 kDa".

Ni et al. is as discussed above in the response to the rejection under 35 USC 102(e).

WO 98/07035 teaches an immunoassay for detection of arthritic degradation products (ADP) in body fluids. One of these ADPs is a 20 kDa fragment of thrombospondin derived from the N-terminus of TSP-1 (see Summary of the Invention). No where does WO 98/07035 teach or suggest a thrombospondin polypeptide weighing 180 kDa or any other weight that is useful as a marker for diagnosing Alzheimer's disease. Thus, WO 98/07035 does not remedy the deficiencies of the teachings of Ni et al.

Bueé et al. used antibodies against thrombospondin to compare its distribution between neurologically normal elderly patients and patients having Alzheimer's disease. This comparison was made post mortem by using immunohistochemical staining of brain tissue

samples. In contrast to Bueé et al., the claimed method of diagnosis of Alzheimer's disease involves testing body fluid obtained from living patients for the presence of a 180 kDa thrombospondin polypeptide. Thus, Bueé et al. do not remedy the deficiencies of the teachings of Ni et al.

The Examiner asserts that Bueé et al. evidence the correlation of the presence of thrombospondin with the occurrence of dementia, even though the correlation was not in blood fluid, this is not a limitation of the claims. However, both the claims as originally filed and as amended herein are clearly limited to samples of body fluid, including blood and/or blood products.

The cited references (Ni et al. US 6,605,592; or in the alternative Ni et al. US PGPub 2002 0068319; Bueé et al. American Journal of Pathology 141(4):783-788 1992; and WO 98/07035) neither separately nor combined teach or suggest all of the claim limitations. Although one of ordinary skill in the art might suspect that thrombospondin is involved with some aspect of Alzheimer's pathogenesis one would have no reasonable expectation of success if attempting to use thrombospondin to diagnose Alzheimer's disease in living patients since one of ordinary skill in the art would not know that the presence of a 180 kDa thrombospondin polypeptide in a sample of body fluid could

distinguish Alzheimer's disease from an age-matched normal physiological state.

Additionally, a reference or combination of references used in support of a rejection under 35 USC 103(a) must enable the claims to which it is applied against in order to properly establish a *prima facie* case of obviousness. Since neither the cited references nor any other prior art teaches and/or suggests a method for diagnosis of Alzheimer's disease by testing body fluid samples for the presence of a 180 kDa thrombospondin polypeptide, the cited references can not enable one of ordinary skill in the art to use the claimed method.

In light of all of the above remarks, Applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of obviousness and further contend that a physician and/or researcher of ordinary skill in this art, having the cited references (Ni et al. US 6,605,592; or in the alternative Ni et al. US PGPub 2002 0068319; Bueé et al. American Journal of Pathology 141(4):783-788 1992; and WO 98/07035) in front of him/her would not have the information and motivation necessary to arrive at Applicants' invention.

Thus, it is respectfully submitted that the cited combination of references fails to reasonably teach or suggest to an artisan of ordinary skill in medicine/biological chemistry the elements of

Applicants' method as specifically set forth in claims 15-17 as presented herein.

Accordingly, Applicants respectfully submit that the claimed method distinguishes over the prior art and respectfully request that this rejection under 35 USC 103(a) now be withdrawn.

CONCLUSION

In light of the foregoing remarks and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,

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ACCELERATED PUBLICATION

Activation of Platelet-transforming Growth Factor β-1 in the Absence of Thrombospondin-1*

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ABSTRACT

Thrombospondin-1 (TSP-1) has been shown to bind and activate transforming growth factor-β1 (TGF-β1). This observation raises the possibility that TSP-1 helps to sequester TGF-β1 in platelet α granules and activates TGF-β1 once both proteins are secreted. Herein, we evaluated the level of active and latent TGF-β1 in the plasma and in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice on two genetic backgrounds (C57BL/6 and 129Sv). The plasminogen activator inhibitor-1/luciferase bioassay and an

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immunological assay were used to determine active and latent TGF-β1. No significant differences were observed in the levels of active and latent TGF-β1 in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice. Active and latent TGF-β1 were significantly increased in the plasma and platelets of C57BL/6 mice as compared with 129Sv mice. In addition, there was an increase of plasma level of latent TGF-β1 in TSP-1 null mice as compared with wild-type mice on the C57BL/6 background but not on the 129Sv background. No active TGF-β1 was observed in the plasma of either TSP-1 null and wild-type mice. These data indicate that TSP-1 does not function as a chaperon for TGF-β1 during platelet production and does not activate significant quantities of secreted TGF-β1 despite a vast excess in the number of TSP-1 molecules as compared with TGF-β1 molecules. Because platelet releasates from TSP-1 null mice contain active TGF-β1, we suggest that other important mechanisms of physiological activation of TGF-β1 probably exist in platelets.

INTRODUCTION

Transforming growth factor-β (TGF-β1, -2, and -3)[1] are mammalian cytokines with a wide range of biological effects (1). They are involved in the regulation of development, proliferation, angiogenesis, inflammation, extracellular matrix production, integrin expression, protease activity, and apoptosis (2). They play a pathologic role in inflammation and fibrotic diseases such as nephrosclerosis (3). Mice null for either, TGF-β1, -2, and -3 do not survive beyond a few days or weeks (4-8). Surviving pups of animals null for TGF-β1 exhibit dysregulated myelopoiesis and a wasting syndrome characterized by an inflammatory response targeting the heart, lung, pancreas, stomach, liver, and striated muscle that has been attributed to an autoimmune process (9, 10). Overexpression of TGF-β1 causes lethality *in utero* or just after birth (11).

TGF-β1 is synthesized by cells in a latent form that must be activated to be recognized by cell-surface receptors and to trigger biological responses (2). Small latent TGF-β1 is a dimeric complex of ~100 kDa, composed of two identical chains in which an amino-terminal 278 amino acid latency-associated peptide (LAP) is noncovalently associated with the carboxyl-terminal 112 amino acid active peptides (12). This latent complex is the product of a single gene. Prior to secretion, LAP is enzymatically cleaved from the active peptide, and the integrity and latency of the secreted complex are presumably maintained via electrostatic interactions (13). Latent TGF-β1 can exist as a large complex in which it is associated with a latent TGF-β1-binding protein (LTBP). LTBP has features in common with

extracellular matrix proteins and targets latent TGF-β1 to the matrix (2). The latency of TGF-β1 is dependent on the presence of LAP; the presence of LTBP is neither necessary nor sufficient for prevention of activation (14).

Physiological mechanisms of activation of TGF-β1 are not well understood (12), although proteolytic processing by plasmin, exposure to reactive oxygen species, and binding to $\alpha_v\beta_6$ integrin may participate in TGF-β1 activation (2, 15). Interaction of latent TGF-β1 with thrombospondin-1 (TSP-1) results in activation of latent TGF-β1 (16-19). TSP-1 is a trimer of disulfide-linked 180-kDa subunits found at high concentrations in platelet α granules and also produced by a number of other cell types (20). It is an adhesive protein with a number of domains available for binding to cell surface or matrix proteins. TSP-1-deficient mice are viable and exhibit subtle abnormalities in development (21). The adult mice exhibit increased inflammatory cell infiltrates and epithelial cell hyperplasia in the lungs, suggesting that TSP-1 is involved in normal lung homeostasis (21). TSP-1 purified from human platelets has been shown to contain TGF-β1 (22). TSP-1 also activates TGF-β1 in cell culture assays when added to endothelial cells. The site in TSP-1 responsible for latent TGF-β1 activation has been localized to the type 1 repeats, especially the $K^{412}RFK^{415}$ sequence located between the first and second type 1 repeats of TSP-1 (16). More recently, Ribeiro *et al.* (23) showed that the TSP-1 sequence KRFK binds LAP through interactions that involve a specific sequence at the amino terminus of LAP ($L^{54}SKL^{57}$). The binding of TSP-1 to LAP appears to induce a conformational change that renders the TGF-β1 active.

Mechanisms controlling conversion of the latent complex to the active state are key regulators of TGF-β1 activity. TSP-1 is the first activator of TGF-β1 shown to function in natural, untreated, nondiseased tissues *in vivo* (24). The previous observations that purified platelet TSP-1 contains associated TGF-β1 (16-19), raises the possibility that TSP-1 activates platelet TGF-β1 or serves as a carrier for TGF-β1 during α granules formation. Here, we evaluated and compared the level of active and latent TGF-β1 in the plasma and in platelet α granules of TSP-1 null and wild-type mice using the plasminogen activator inhibitor-1/luciferase bioassay (25) and the commercial TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Boston, MA). We observed that (i) there is no active TGF-β1 in the plasma of both TSP-1 null and wild-type mice, (ii) there is an increase in the level of plasma latent TGF-β1 in TSP-1 null C57BL/6 mice compared with wild-type, and (iii) there is no significant difference in the level of active and latent TGF-β1 in platelets from TSP-1 null and wild-type mice. Despite the absence of TSP-1 in TSP-1 null mice, active TGF-β1 is observed in the platelet releasates. These data suggest that TSP-1 is not the only physiological activator of TGF-β1 in platelet

α granules and that TSP-1 does not play a role of carrier for TGF-β1 during platelet biosynthesis.

EXPERIMENTAL PROCEDURES

Animals-- TSP-1 null animals were generated by homologous recombination in 129Sv-derived ES cells, as described previously (21).

Preparation and Stimulation of Mouse Platelets-- Mice were anesthetized with 2.5% avertin. Blood was drawn by periorbital insertion of a heparinized capillary tube (~1.5 cm in length). Blood from five to eight TSP-1 null and wild-type (C57BL/6 or 129Sv) mice was drawn into tubes containing acid-citrate dextrose and pooled separately. Platelets were isolated by differential centrifugation and washed in pH 6.5 buffer containing 0.102 M NaCl, 3.9 mM K_2HPO_4, 3.9 mM Na_2HPO_4, 22 mM NaH_2PO_4, and 5.5 mM glucose. The platelets were resuspended in 15 mM Tris-HCl (pH 7.6), 0.14 M NaCl, 5 mM glucose, and 2 mM $CaCl_2$. The platelet counts were determined and platelet concentration was adjusted to 1.8×10^5 platelets/μl. Platelets were activated with human thrombin (0.5 unit/ml; Sigma) for 3 min under constant stirring. Platelet aggregates were centrifuged at 3,000 × *g*, and the releasates of thrombin-stimulated platelets were collected and stored at −20 °C for further quantification of total and active TGF-β1 by both the TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay (25). In other experiments, thrombin-activated platelets are treated with 10 mM EDTA for 10 min before the collection of the releasate of thrombin-stimulated platelets.

Quantification of Total and Active TGF-β1 by ELISA Kit-- Total and active TGF-β1 concentration in the plasma and in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice were assayed by a sandwich TGF-β1 ELISA kit (Genzyme, Boston, MA) according to the manufacturer's specifications. Plasma and supernatant of thrombin-treated platelets were thawed and divided into two pools. In the first pool, acid activation was required to convert latent to active TGF-β1 and to record detectable levels of total TGF-β1. By contrast, no acid activation is used in the second pool; the TGF-β1 levels in the samples are therefore representative of active TGF-β1 in the plasma or in the supernatant of thrombin-treated platelets. Plasma (10 μl) and supernatant of thrombin-treated platelets (50 μl) were added to sample diluent with or without 1 N HCl for 60 min at 4 °C followed by neutralization with 1 N NaOH if samples were activated with HCl. Samples were plated on microtiter plates coated with anti-TGF-β1 antibody and incubated at 37 °C for 60 min. After vigorous washing,

wells were incubated with a second biotin-conjugated anti-TGF-β1 antibody, and the peroxidase reaction was initiated. A standard curve was constructed using serial dilutions of human TGF-β1 (Genzyme) as standard. TGF-β1 levels in samples were compared with known standards and read as nanograms per milliliter.

Quantification of Total and Active TGF-β1 by the Plasminogen Activator Inhibitor-1/Luciferase Bioassay-- Total and active TGF-β1 concentration in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type mice were assayed using the plasminogen activator inhibitor-1/luciferase (PAI/L) assay, first described by Abe *et al.* (25). This assay is based on the ability of TGF-β1 to induce plasminogen activator inhibitor-1 (PAI-1) expression in mink lung epithelial cells (MLECs) transfected with a construct containing a truncated PAI-1 promoter fused to a firefly luciferase reporter gene. Transfected MLECs were a generous gift from Dr. D. B. Rifkin (New York University Medical Center). Cells were maintained in high glucose (4,500 mg/liter) Dulbecco's modified Eagle's medium (Life Technologies, Inc., Paisley, UK) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 200 μg/ml Geneticin (G418-sulfate) (Calbiochem-Novabiochem Ltd., Nottingham, United Kingdom (UK)). Cells were cultured in a humid atmosphere at 37 °C, 5% CO_2, and passaged maximally 30 times. As described above, the supernatants of thrombin-treated platelets were divided into two pools. This time, however, the first pool was diluted in serum-free Dulbecco's modified Eagle's medium containing 0.1% pyrogen-poor bovine serum albumin (Pierce & Warriner UK Ltd., Chester, UK) and heat-activated for 10 min at 80 °C. As before, the second pool remained untreated so as to measure the amount of active TGF-β1 present. MLECs were trypsinized and washed and the cell density adjusted to 1.6×10^5 cells/ml before plating 100 μl/well into a 96-well tissue culture plate (Falcon, Becton Dickenson, Oxford, UK). Cells were incubated for 3-4 h to allow for optimal attachment to the plastic. Following aspiration of the growth medium from the attached cells, 100 μl of the sample was added in triplicate. Cells and samples were then incubated for 14-16 h at 37 °C, 5% CO_2. Following incubation, all wells were checked microscopically for cell viability before washing twice with 100 μl of phosphate-buffered saline. Cells were then lysed using 100 μl/well of 1 × lysis buffer (Promega, Southampton, UK) and incubated with agitation at room temperature for 20 min. Forty-five microliters of the cell lysates were transferred to an opaque Microlite^{TM} 1 Microtiter® read plate (Dynex Technologies Ltd., West Sussex, UK). Lysates were analyzed for luciferase activity using an MLX Luminometer (Dynex Technologies Ltd.) following the injection of 110 μl/well of substrate solution (20 mM tricine, 1.07 mM $Mg(CO)_3Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 750 μM

ATP, and 800 μM luciferin (Promega, Southampton, UK)). The flash of light obtained upon mixing the lysates with the substrate was recorded as relative light units. The mean values of the triplicates were then converted into concentrations of TGF-β1 in picograms per milliliter using a standard curve obtained with human recombinant TGF-β1 (R & D Systems, Abingdon, UK).

Statistical Analysis-- Data are expressed as the mean ± S.E. Statistical evaluation of the data was performed using the unpaired *t* test, considering *p* values <0.05 as significant.

RESULTS AND DISCUSSION

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Since TGF-β1 binds to TSP-1 with high affinity (16-19), we hypothesized that TSP-1 may function as a carrier protein for TGF-β1. To test this hypothesis, we assayed the quantity of TGF-β1 that is secreted from wild-type and TSP-1 null platelets in response to thrombin. As shown in Fig. 1, the level of total TGF-β1 is equivalent in the supernatants from the wild-type and TSP-1 null platelets. We have confirmed this result by employing two different assays for TGF-β1 and two different strains of TSP-1 null mouse. In our hands, the bioassay consistently gives higher values than the TGF-β1 ELISA kit; however, the relative levels of active TGF-β1 as compared with total TGF-β1 are consistent between the two assays.

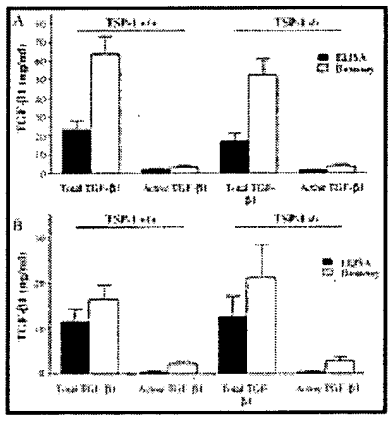

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Fig. 1. Active TGF-β1 is present in the supernatant of thrombin-treated platelets from TSP-1 null mice. Mice platelets from TSP-1 null and wild-type mice were activated with 0.5 unit/ml human thrombin for 3 min, platelet suspensions were centrifuged, and platelet supernatants were removed. Total and active TGF-β1 were assayed in the supernatant of thrombin-treated platelets from TSP-1 null and wild-type C57BL/6 (*A*) and 129Sv (*B*) mice by a TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay. Results are expressed as the means ± S.E. of duplicate determinations of four to six separate experiments (*ELISA*) and three to four separate experiments (*Bioassay*).

TSP-1 reportedly binds to the platelet membrane after secretion when calcium is present (26). Thus,

TGF-β1 that is complexed with TSP-1 may also become associated with the platelet membrane. If this is the case, then the TGF-β1 levels that we measured in the supernatant of the thrombin-treated wild-type platelets may be anomalously low. To determine whether this is the case, we treated the platelets with 10 mM EDTA after thrombin treatment to remove the TSP-1 from the platelet membrane (Fig. 2). Treatment of the platelets with EDTA resulted in an increase in the level of TGF-β1 in the supernatants of both the wild-type and the TSP-1 null platelets (Fig. 2). Thus, while there appears to be a calcium-dependent mechanism for the association of TGF-β1 to the platelet membrane, it does not require TSP-1. It is known that LAP contains an arginine-glycine-aspartate (RGD) sequence that might function to localize latent cytokine to the cell surface by binding integrins (2). Grainger *et al.* (27) reported indirect evidence that the RGD sequence in platelet-derived latent TGF-β1 may be recognized by platelet integrins. Like several other matrix proteins, human LTBP also contains an RGD sequence; however, there are no reports that this sequence serves as an integrin ligand (2). In wild-type mice, LAP can remain associated with the TSP-1/TGF-β1 complex without inhibiting the activity of TSP-1-associated TGF-β1 (23).

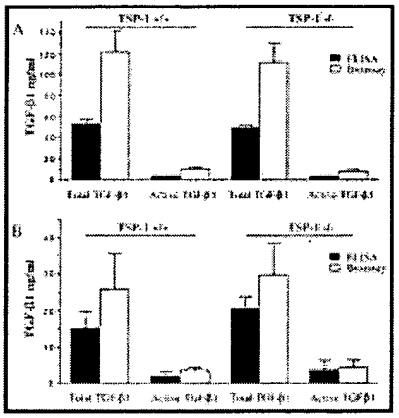

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Fig. 2. Treatment of platelets with EDTA increases the level of TGF-β1 in the supernatants of both the wild-type and the TSP-1 null platelets. Mice platelets from TSP-1 null and wild-type were activated with 0.5 unit/ml human thrombin for 3 min and treated with 10 mM EDTA for 10 min. Platelet suspensions were centrifuged and platelet supernatants were removed. Total and active TGF-β1 were assayed in platelet releasates from TSP-1 null and wild-type C57BL/6 (*A*) and 129Sv (*B*) mice by a TGF-β1 ELISA kit and the plasminogen activator inhibitor-1/luciferase bioassay. Results are expressed as the means ± S.E. of duplicate determinations of three to five separate experiments (*ELISA*) and three to four separate experiments (*Bioassay*).

Surprisingly, we have found that the absence of TSP-1 has no effect on the level of active TGF-β1 in the supernatant of thrombin-treated platelets (Figs. 1 and 2). In the lung, pancreas, and liver, TSP-1 null mice exhibit pathologies that are similar to, although generally not as severe as, TGF-β1 null mice (21, 24). The abnormalities include epithelial cell hyperplasia and focal inflammation. Treatment of the TSP-1 null mice with the TGF-β1-activating peptide KRFK normalizes these abnormalities in the lung and pancreas (24). Furthermore, treatment of wild-type mice with a peptide (LSKL) that inhibits the ability of TSP-1 to activate TGF-β1 induces lung and pancreas pathologies similar to those seen in

the TSP-1 null mice (24). These data indicate that constitutive activation of TGF-β1 by TSP-1 contributes to epithelial homeostasis in some organs. It remains possible that TSP-1 is an important activator of TGF-β1 in platelets from wild-type mice and that an alternative pathway is up-regulated in the absence of TSP-1. Since active TGF-β1 is present in the supernatant of thrombin-treated TSP-1 null platelets, alternative mechanisms for TGF-β1 activation must be present in platelets. These mechanisms may involve the association of TGF-β1 with another protein that is present in the α-granules or shed from the platelet membrane after activation. Since the platelets are removed shortly after thrombin-treatment, it is unlikely that the activation of TGF-β1 is due to the release of a constituent of the lysosomal compartment. It is also unlikely that the amount of thrombin used or the duration of exposure to thrombin would be sufficient to activate significant levels of TGF-β1. In fact, human thrombin (0.1, 0.5 and 1 unit/ml) added to both serum and platelet releasate samples for 30 min did not increase the amount of active TGF-β1, as compared with non-thrombin-treated samples, demonstrating that thrombin was not responsible for the active TGF-β1 detected in our assays (data not shown). An alternative interpretation of the data is that TSP-1 is not a significant activator of TGF-β1 in platelets. In wild-type platelets, the majority of TGF-β1 is inactive despite a vast excess in the number of TSP-1 molecules as compared with TGF-β1 molecules (12, 28). Since the type 1 repeats appear to be important for protein-protein interactions in general, it is possible that TGF-β1 binding to TSP-1 is inhibited by the presence of another protein. It is also possible that post-translational modifications of TSP-1 that occur in megakaryocytes inhibit TGF-β1 binding. The data clearly show that coexpression of TSP-1 and TGF-β1 does not necessarily mean that TGF-β1 will be activated via a TSP-1-dependent mechanism. Because TSP-1 and TGF-β1 are stored and secreted together from platelet α granules, and because active TGF-β1 has a short half-life, an independent spatial and temporal mechanism for regulating TGF-β1 activation may be necessary. This mechanism may not be active in epithelial cells because TGF-β1 and TSP-1 are constitutively secreted and a basal level of activated TGF-β1 is maintained.

We also assayed the levels of TGF-β1 in the plasma of wild-type and TSP-1 null mice to determine whether there is a systemic increase in TGF-β1 levels that might compensate for the lack of a TSP-1-dependent activation mechanisms that exist in epithelial tissues (21, 24). A statistically significant ($p < 0.01$) increase in the total TGF-β1 was observed in the plasma of the TSP-1 null mice as compared with wild-type mice on the C57BL/6 background (Table I). By contrast, no difference was observed in the plasma levels of TGF-β1 in the TSP-1 null mice and the wild-type mice on the 129Sv background. No active TGF-β1 was found in the plasma of either TSP-1 null and wild-type mice. Free TGF-β1 can

interact with and be inactivated by a number of soluble or matrix molecules, including α_2-macroglobulin, decorin, betaglycan, and fucoidan (29, 30). Furthermore, TGF-β1 in plasma is found almost exclusively bound to α2-macroglobulin and presumably represents activated TGF-β1 that will be cleared by the liver (31). The physiological mechanisms for the increase in the level of latent TGF-β1 in TSP-1 null C57BL/6 mice is currently unknown.

Table I

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Plasma TGF-β 1 in C57BL/6 and 129Sv mice

TGF-β1 levels in the plasma of C57BL/6 and 129Sv mice were determined by a TGF-β1 ELISA kit. Results are expressed as the means ± S.E. of two duplicate determinations of 22-23 separate experiments for C57BL/6 mice and 22-26 separate experiments for 129Sv mice.

In this study, we have shown that the levels of latent and active TGF-β1 in the supernatant of thrombin-treated platelets are equivalent in the presence or absence of TSP-1. Thus, TSP-1 does not appear to function as a chaperon for TGF-β1 during platelet production. The lack of correlation between TSP-1 expression and the level of active TGF-β1 indicates that (i) the ability of TSP-1 to activate TGF-β1 is inhibited in wild-type mice and (ii) alternative mechanisms for activation of TGF-β1 are present in platelets. Elucidation of the mechanisms underlying each of the observations will have important implications for the regulation of TGF-β1 activation *in vivo*.

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FOOTNOTES

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ABBREVIATIONS

The abbreviations used are: TGF-β1, transforming growth factor-β; LAP, latency-associated peptide; LTBP, latent TGF-β1-binding protein; TSP-1, thrombospondin-1; ELISA, enzyme-linked immunosorbent assay; MLEC, mink lung epithelial cell.

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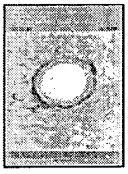

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